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Probing the uterine microenvironment through Systems Biology approaches

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Abstract

In cattle, the developing preimplantation conceptus depends solely on its surrounding environment for supplying nutrients and growth stimuli. Also, throughout gestation, it is during the preimplantation stage of gestation that most conceptus mortality is observed. Thus, one could rationalize that the composition of the uterine environment plays a major role on conceptus survival. However, the biochemical composition of the uterine environment is only just starting to be deciphered and conditions associated with optimal conceptus development are virtually unknown. In this review we attempt to briefly review and discuss issues associated with sampling and analyzing the uterine environment. We suggest that modern, holistic approaches such as Systems Biology are necessary to characterize the uterine functions associated with pregnancy success in cattle.

Keywords: endometrial biopsy, histotroph, systems biology, uterine flushing, uterine receptivity.

Introduction

In cattle, a surge of GnRH is associated with the beginning of estrus behavior. Approximately 30 h later, ovulation of the preovulatory follicle occurs followed by delivery of the oocyte to the infundibulum of the oviduct. Movement of the oviductal cilia and the flow of oviductal fluid bring the oocyte to the ampula, which is the site of fertilization. Successful fertilization will trigger the formation of a zygote and cell divisions will initiate. Early embryo development takes place in the oviduct. Between days 4 and 5 after estrus the early embryo enters the uterus, where the remaining of gestation occurs. Implantation takes place at about day 20 to 21. The preimplantation uterine development although short, is an eventful process. Embryos grow and differentiate from a microscopic spherical shape morula to a fully elongated filamentous conceptus that spans the length of both uterine horns. An interesting feature of this process is that there is no attachment of the conceptus to the maternal tissues and the conceptus is considered to be "free floating". Thus, maternal supply of molecules critical to conceptus growth and survival, such as nutrients and growth factors, must be via secretions to the uterine lumen. Secretions and fluids accumulating in the uterine lumen are called collectively "histotroph".

In cattle, preimplantation conceptus mortality is around 30% (Diskin and Morris, 2008). The working hypothesis of this paper is that composition of the histotroph during the preimplantation period is associated with pregnancy success. Supporting evidence for this concept includes de following: 1) because there is no attachment of the conceptus to the endometrium, there are no other sources of molecules to the conceptus but the histotroph (Spencer and Gray, 2006); 2) throughout pregnancy, conceptus mortality is highest during the preimplantation period (Diskin and Morris, 2008); 3) conceptus genetic aberrations cause mortality soon after fertilization (Diskin and Sreenan, 1980), thus mortality is generally associated with environmental causes (i.e., the uterine environment, that is, the histotroph). Despite the obvious relevance of understanding the association between composition of the histotroph and embryo survival, very little attention has been given to this area of research. Specifically, studies are necessary to interrogate the biochemical composition of the histotroph. This includes the identification and the quantification of concentrations of molecules of different biochemical classes. Most importantly, such a survey of the uterine environment must be conducted in a comprehensive fashion. Instead of the usual reductionist approach, in which concentrations of individual molecules are measured and biological relevance is inferred from limited information, novel, holistic approaches to understand biological complexity have been proposed (Spencer *et al.*, 2008). Through Systems Biology, or High Density Biology (HDB) approaches, data obtained from "omics" techniques are analyzed together, using multivariate statistics and biochemical data banks (D'Alexandri, 2010). Biochemical pathways enriched in the tissue of interest are identified. Targets for understanding and

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manipulating that tissue's biology are specified and can be further researched.

Studies using Systems Biology approaches to investigate preimplantation uterine biology are non-existing in cattle. To test our working hypothesis it is critical that the composition of the histotroph is determined. Specifically, information on differences in the composition of uterine environments associated with pregnancy success and failure must be obtained. This brief review addresses how to sample the uterine environment and how to analyze the uterine environment on an attempt to gain insight on the uterine functional contributions to preimplantation conceptus survival.

Sampling the uterine environment

To determine the composition of the uterine environment the obvious first step is to sample it. Histotroph samples represent the most direct, real-time assessment of the environment in which the conceptus is developing. The uterine environment could be assessed indirectly by obtaining endometrium samples, which is the tissue that secretes molecules into the uterine lumen to produce the histotroph. In both situations, sampling the uterine environment during preimplantation is intrinsically challenging for several reasons. First, there is no direct method to precisely detect the presence of a conceptus that early on gestation, except by flushing the uterus and visualizing the conceptus in the uterine flush. The implication is that presence of a conceptus in the flush does not guarantee viability and postimplantation survival is not possible to measure. Second, if postimplantation viability needs to be verified, sampling during the preimplantation period must interfere minimally with conceptus development. This is difficult, because probing the uterine environment, through flushings or tissue biopsies, is an invasive procedure and the odds of disturbing pregnancy are high. Results from studies using these sampling techniques are described below.

Under good conditions of health, nutrition and husbandry there are no phenotypic characteristics that distinguish females that have uterine environments that are more or less adequate to maintain a pregnancy. Thus, studies have relied on experimental models to generate uterine environments of contrasting quality regarding embryo receptivity. Such models are based on classical knowledge of ovarian steroids on uterine function. Typically, researchers manipulate ovarian function or supplement ovarian steroids to indirectly affect uterine function. Specifically, in beef cattle, manipulations include the stimulation of growth of the preovulatory follicle and the stimulation of growth of the corpus luteum after ovulation to generate a uterine environment associated with higher fertility, and vice-versa.

In this section, models to manipulate uterine function, sampling uterine secretion, and sampling uterine tissue will be discussed.

In vivo models to manipulate uterine function

The contemporaneity of postovulatory rise of plasma progesterone concentrations with the establishment and maintenance of early embryonic development, followed by evidence indicating the need of an active corpus luteum for pregnancy maintenance (Estergreen *et al.*, 1967) draw the attention to progesterone as a *pro-gestation* hormone and obvious target for manipulation in studies aiming to improve pregnancy success. Current models studying uterine function in response to postovulatory progesterone concentrations have mainly focused on pharmacological manipulation of progesterone concentrations during the early postovulatory phase.

Experiments designed by Dr. Fuler Bazer's and Dr. Thomas Spencer's group to investigate uterine gene expression and conceptus development and survival in response to progesterone have focused on exogenous progesterone supplementation and inhibition of progesterone activity by its receptor antagonist, RU486. Their approach involved daily injections of 25 mg of progesterone starting as early as day 1.5 and extending up to days 6, 9 or 12 postmating in ewes. Progesterone supplementation resulted in higher progesterone concentrations until day 12 in comparison to corn oil-treated control. Additionally, progesterone supplemented ewes produced hatched blastocysts that were 220% greater on day 9 and that were all elongated by day 12 versus spherical to tubular conceptuses in corn oil-treated ewes (Satterfield *et al.*, 2006).

In cattle, Dr. Patrick Lonergan's group has also focused on pharmacological manipulation of postovulatory progesterone concentrations by using two different approaches. First, Carter *et al.* (2008) inserted a progesterone releasing-intravaginal device (PRID) in pregnant and non-pregnant heifers on day 3 postestrus. Heifers receiving progesterone supplementation had higher progesterone concentrations from days 3.5 to 6.5, reaching approximately 5 to 6-fold higher concentrations than those from heifers not being supplemented. Furthermore, conceptuses from supplemented heifers were significantly more elongated on days 13 and 16 postestrus than those from not supplemented animals. No difference in conceptus development on days 5 and 7 and no negative effect on corpus luteum development were observed (Carter *et al.*, 2008). In addition to generating animals with higher postovulatory progesterone concentrations, Dr. Patrick Lonergan's group also developed a model to reduce progesterone concentrations during early diestrus in beef cows. Beltman *et al.* (2009) used 3 injections of prostaglandin F2α analog every 12 h from days 3 to 4 or 4.5 to reduce progesterone concentrations in comparison to untreated control heifers. Although sufficient to reduce corpus luteum secretory capacity both 3 and 4 injections of prostaglandin F2α induced regression of the corpus

luteum in 30 and 60% of the animals, respectively (Beltman *et al.*, 2009).

The aforementioned models to study the influence of progesterone on uterine molecular profile and conceptus development are widely accepted and have generated a significant amount of relevant information to the scientific community. However, questions may arise if one is to make a parallel between such models and the physiological cyclic changes that compose the estrous cycle. Although exogenous supplementation of progesterone is successful in inducing an increase in plasma progesterone concentrations it also causes a sudden and marked rise in concentrations of this ovarian steroid, a condition that is unparalleled under physiological conditions. Secondly, it has been demonstrated that early exposure to high progesterone concentrations impairs CL function and induces premature luteolysis, though according to Carter *et al.* (2008) this response has not been observed in their studies (Carter *et al.*, 2008). Similarly, depression of luteal function by PGF analog treatment during metaestrus is not a normally occurring process in the physiology of the estrous cycle or pregnancy, which may be permanently detrimental to the maintenance of CL secretory capacity.

An alternative approach to control postovulatory progesterone concentrations by manipulating the preovulatory follicle has been reported in 1991 by Milvae *et al.* (1991), which by means of aspiration of the preovulatory follicle 18 h postestrus reduced the corpus luteum capacity of producing progesterone in comparison to no-aspiration control cows. Interestingly, cows that had the preovulatory follicle aspirated and follicular fluid subsequently returned into the follicle had similar progesterone concentrations in comparison to control cows (Milvae *et al.*, 1991). More recently, O'Hara *et al.* (2012) used aspiration of the preovulatory follicle to assess corpus luteum development, progesterone production, and embryonic development in unstimulated and superovulated cows. Follicular aspiration of the dominant follicle just prior to ovulation reduced corpus luteum diameter from days 6 to 13, as well as progesterone from days 7 to 13. On day 14, at slaughter, corpora lutea of aspirated follicles had reduced weight, diameter and area, and length and area of conceptus from aspirated cows were also reduced (O'Hara *et al.*, 2012).

Another aspect that is not contemplated by the currently available models to modulate uterine function refers to the sequential, gradual and timely exposure of the uterus to the ovarian steroids, estradiol and progesterone. Supported by data showing positive correlations between preovulatory follicle diameter and fertility as well as postovulatory progesterone concentrations (Meneghetti *et al.*, 2009; Peres *et al.*, 2009; Pfeifer *et al.*, 2009; Sa Filho *et al.*, 2010) our laboratory took a holistic approach to generate and characterize two distinct groups of animals commonly occurring in field conditions: 1) cows ovulating smaller follicles and therefore developing smaller corpora lutea and producing less progesterone (low postovulatory progesterone group; LP), and 2) cows ovulating larger follicles, developing larger corpora lutea and producing more progesterone (high postovulatory progesterone group; HP). Preovulatory follicle diameter, which is directly correlated to estradiol concentrations during proestrus and estrus (Lopes *et al.*, 2007), and the postovulatory gradual rise in progesterone concentrations compose what will be herein called the periovulatory endocrine milieu.

The implementation of this model was based on the basic reproductive physiology principle of negative feedback on LH pulse frequency exerted by progesterone. Therefore, growth of the dominant follicle was manipulated under the critical premise that all animals had to have a PGF-responsive corpus luteum at the beginning of the hormonal protocol (day -10; progesterone device insertion). The first critical step to generate two groups of animals with distinct periovulatory endocrine milieus was to modulate progesterone concentration during dominant follicle growth; therefore, cows from the HP group received a PGF injection at progesterone device insertion, whereas LP cows did not (unpublished data). This manipulation was expected to stimulate dominant follicle growth rate and to increase the diameter of the preovulatory follicle in cows of the HP group. The second critical step of our model aimed to manipulate the interval between suppression of plasma progesterone concentrations (via an injection of PGF) and the administration of the ovulatory stimulus (i.e. GnRH analog injection), which we called the proestrus period. The rationale was that follicles from cows of the HP group should have a longer proestrus interval. Thus, follicles would have more time to grow under basal progesterone concentrations, reaching a larger preovulatory diameter. This model was successful in generating distinct periovulatory endocrine milieus as preovulatory follicle diameters were 13.8 mm and 11.7 mm, and progesterone concentrations on day 6 post-estrus were 3.9 ng/ml and 2.4 ng/ml for HP and LP groups, respectively. Moreover, ovulations were within 24 and 36 h post-GnRH. Furthermore, molecular evidence indicated that the ovarian manipulation and resulting distinct periovulatory endocrine milieus were sufficient to alter endometrial gene expression according to the expected physiological response to differential progesterone concentrations, namely downregulation of progesterone and oxytocin receptor genes in cows with higher progesterone concentrations (unpublished data).

Sampling uterine secretions

The collection of uterine secretions *in vivo* can be achieved through conventional techniques similar to uterine flushings to recover embryos from donor cows. Briefly, a Foley catheter is inserted in the reproductive tract of the animal through the cervix, positioned in the

uterine location from where a sample is needed. Then, one of several possible media is injected through the catheter and recovered for later analyses. Issues such as the volume of medium injected, which uterine horn is flushed (i.e., contralateral or ipsilateral to the ovary containing a CL), the physiological status of the animal (anestrus, cyclic or pregnant) and the stage of conceptus development must be taken in consideration. This is because composition of flushings and post-flushing embryo survival will be affected by such factors.

Another way to obtain uterine secretions is by flushing the uterus post-mortem. This can be done in the laboratory in a more controlled way, compared to the *in vivo* collection. However, because contributions to the histotroph composition are also from the circulation, such as from serum transudates, composition *in vivo* is expected to be different than composition post-mortem. To test this, Niemeyer *et al.* (2007) compared protein composition of uterine flushings obtained *in vivo* or post-mortem from cyclic and pregnant cows on day 17 postestrus using 1-D electrophoresis and coomassie blue staining. From the twenty protein bands identified, the optical density (an indirect measure of protein content) of twelve bands was different comparing flushings obtained *in vivo vs*. post-mortem. In contrast, the low sensitivity method to quantify proteins used detected only one protein band that was different between pregnant and cyclic cows (Niemeyer *et al*., 2007).

On a recent experiment conducted in our laboratory, we aimed to measure the effects of collecting a uterine flush or a uterine biopsy on pregnancies per AI measured 30 days after AI. Cyclic, multiparous nelore cows (n = 150) were timed-inseminated after a conventional estradiol benzoate-progestagen synchronization protocol (Sa Filho *et al.*, 2011). Six days after AI, cows were homogeneously divided according to parity, body weight and body condition score in three groups. The control group was not submitted to any procedures, the flushing group was submitted to a 20 ml flushing of the uterine horn contralateral to the ovary bearing the CL and the biopsy group was submitted to collection of an endometrial biopsy of the horn contralateral to the CL. Pregnancy per AI was 32.5% for the control group, 25% for the flushing group and 25.6% for the biopsy group. As expected, there was a reduction in pregnancies per AI for both the flushing and biopsy groups compared to the control animals. However, such reduction was not catastrophic to pregnancy. It was concluded that it was possible to sample the uterus during early gestation without necessarily causing loss of that gestation and cows have different sensitivities to uterine sampling during gestation.

Sampling uterine tissue

An indirect way to access the composition of the uterine environment is to study the composition of the endometrium. Much of the histotroph are molecules synthesized and secreted from glandular and luminal endometrial epithelial cells. For example, one may be interested in measuring the concentration of specific endometrial mRNAs and proteins that are suspected to contribute to histotroph composition. In this context, sampling the endometrium becomes relevant, and this may be done *in vivo* using conventional biopsy techniques. Again, one may be concerned with the effects of collecting an endometrial biopsy on an ongoing pregnancy. As mentioned above and similar to uterine flushings, our results indicate that collecting biopsies 7 days after estrus caused a 21% reduction in pregnancies per AI, which is considered not to be exaggerated. Rhoads *et al.* (2008) collected biopsies from both uterine horns of lactating dairy cows at estrus, 3 days before estrus and 4 days after estrus. Although they did not have a control group not submitted to biopsy, average conception rate was about 50%, which is considered normal for dairy cows. This again supports the concept that, at least early in the preimplantation period, collecting a biopsy from the uterus may not significantly interfere with pregnancy.

It should be emphasized however, that sampling the uterus after conceptus elongation (i.e., after day 15 of pregnancy) will increase the risk of affecting pregnancy outcome more severely. This is because physical damage to the conceptus is likely.

Analyzing the uterine environment

Transcriptomics analyses

Traditionally, gene expression experiments have been conducted by the use of techniques that require *a priori* selection of candidate genes, limiting, therefore, the identification of metabolic and signaling pathways and the investigation of interactions between gene networks (Roy *et al.*, 2011). The availability of genome-wide information for an increasing number of species enables the identification of gene sequences and the use of the chemical and physical interactions of complementary nucleic acid sequences to develop the microarray technology. Approximately 30 years ago the first microarray study, looking at screening colon tumors in mice, was published (Augenlicht and Kobrin, 1982). In 2001, Yao *et al.* published the first cDNA microarray study in cattle, focusing on approximately 700 unique genes, and one year later scientists at University of Illinois published a manuscript reporting the first large-scale bovine microarray containing 3,800 elements, available for functional genomics (Yao *et al.*, 2001; Band *et al.*, 2002). In addition to the custom-made arrays, several years later, around the year of 2004, the first commercial high-density bovine oligo microarray was made available by Affymetrix Inc. Microarrays consist of a large number of DNA sequences attached to a solid surface that serve

as substrate for the hybridization of fluorescently labeled complementary DNA. Upon recognition of complementary sequences on the array by the population of cDNA-converted RNA species, the respective probe will have its fluorescent signal read and transformed to intensity values by an appropriate scanner. Although the technique itself as well as normalization procedures and statistical analyses are well established, numerous limitations have been identified along the years, namely need for data normalization (spatial artifacts and systematic biases), appropriate statistical analyses to control for multiple tests and reduce false-positive results, high background hybridization levels, difference in probe hybridization properties and dye biases, limited ability to detect low abundance signals and high abundance transcripts, limited ability to detect splice variants, and transcriptional profile is limited to the number of probes in the array (Roy *et al.*, 2011).

The recent development of novel high-throughput DNA sequencing allowed for a major step forward in regards to global transcriptional profiling studies. Based on deep-sequencing methods, the RNA sequencing technique, or RNA-seq, sequences a population of cDNA-converted RNA in a high-throughput manner. Most important, whole transcriptional profile does not depend on current genome information. The resulting sequenced fragments or reads need to be aligned and mapped to a reference genome or transcriptome, or can be assembled without a reference, then generating a transcription profile indicating the transcriptional structure as well as expression values for each gene (Wang *et al.*, 2009). RNA-seq technology has not reached the technical status of microarrays as far as data processing and analysis and further improvements are necessary, however numerous significant advantages solidify the technique as the next generation transcriptomics tool. Some of these advantages are the sensitivity for less abundant transcripts, wider dynamic range to determine transcript abundance, allele-specific expression, quantitation and identification of alternative splicing, not limited to sequences present in an array platform, therefore capable of detecting novel transcripts (particularly important for non-model species with unavailable or incomplete genome information), high resolution (single base-pair), low background noise and low RNA amount required (Wang *et al.*, 2009). On the other hand, as any new technique numerous challenges exist, such as difficulties in controlling bias introduced by manipulations during cDNA library production (i.e. RNA or cDNA fragmentation and cDNA amplification steps), bioinformatics challenges, particularly regarding storage, retrieval and processing of large datasets, which are intrinsic problems of omics approaches, as well as mapping, assembling and alignment of reads to genomes. Additionally, cost may be a limitation as greater genomes associated with greater transcriptomes imply the need for more sequencing depth for adequate coverage, having direct implications in costs (Wang *et al.*, 2009).

A significant body of literature based on genome-wide transcriptional profiling has provided major relevant information regarding uterine biology and *in vivo* conceptus development, either in response to progesterone or in regards to temporal-associated changes during cycle and/or pregnancy as well as pregnancy status. Not surprisingly, Dr. Patrick Lonergan's group is a major contributor to this advances. Using microarray analyses Clemente *et al.* (2011) have identified 465 genes that are likely involved in the transition from blastocyst to ovoid conceptus in cattle (Clemente *et al.*, 2011). Additionally, it has been determined that 194 genes are differentially expressed when embryos from heifers that received progesterone supplementation are compared to embryos from heifers that were not supplemented (Carter *et al.*, 2010). The same group determined that endometrial gene expression is also altered in response to exogenous progesterone supplementation, which also advances endometrial expression profile by altering time and duration of gene-specific expression (Forde *et al.*, 2009). In agreement with those results, Satterfield *et al.* (2009) have also identified the differential endometrial gene expression in response to progesterone supplementation and day of pregnancy in sheep (Satterfield *et al.*, 2009). Recently, our laboratory performed a microarray study (unpublished data) aiming to compare global endometrial gene expression between cows with distinct periovulatory endocrine milieus (i.e. smaller preovulatory follicles and lower postovulatory progesterone concentrations versus larger preovulatory follicles and greater postovulatory progesterone concentrations). Gene ontology analysis of the data revealed the differential expression of genes associated with regulation of biomolecules (secretion of amino acid, amino acid metabolism and degradation of lipids), blood vessel structure (adhesion of endothelial cells and morphology of vessel) and attraction and migration of immune cells (migration of cells, infiltration of macrophages, homing of mononuclear leukocytes, chemotaxis of mononuclear leukocytes, immune response). From the latter biological function several genes had their differential expression confirmed by quantitative PCR, namely CXCL16, PLA2G10, ICAM1, DPP4, CD34, BMP4 and LCP1, providing further support for a relevant role of the immune cells in the local endometrial environment during the first week postestrus.

The state of the art RNA sequencing technology has also reached studies of uterine biology. Studies by Mamo and coworkers have identified, on day 16 conceptus and pregnant endometrium, 133 conceptus ligands (46 conceptus-specific) and 121 endometrial ligands (34 endometrium-specific), which interact with corresponding receptors in the uterus and conceptus,

respectively. Furthermore, they have identified 9 groups of genes clustered by expression profile of bovine conceptus on days 7, 10, 13, 16 and 19, of which two clusters were upregulated on days 16 and 19, thus potentially involved with maternal recognition and implantation (Mamo *et al.*, 2011, 2012).

Proteomics analyses

Proteomics is generally defined as the identification and quantification of all the expressed proteins of a biological sample, such as cells or embryos, with the goal of understanding their functions, their interactions, and their contribution to biological processes (Ferreira, 2011). The proteomics era is coincident with significant developments in the mass spectrometry field (MS), as well as fast bioinformatics tools, database search engines, and fulfillment of genome sequencing efforts. Proteomics involves not only the identification of gene products and their abundances, but also the use of protein interactomes for analysis of protein complexes, protein-protein interaction networks, and the dynamic behavior of the networks as a function of time or experimental condition.

Since 1975 with the O'Farrell high-resolution two-dimensional electrophoresis of proteins (2D) technique (O'Farrell, 1975), the separation of proteins in complex mixture could be accomplished. Analyzing the digitalized images of the pre-labeled or stained 2D gels, it was possible to quantify and identify different protein spots in different samples. However, the identity of most of the proteins remained unknown. With the advent of genome and the development of new protein ionization techniques coupled to mass spectrometry (MS), as Electrospray ionization (ESI) and Matrix Assisted Laser Desorption Ionization (MALDI), MS became the major driving force in the field of proteomics. Traditional MS-based proteomics strategies employ mostly MALDI - time of flight mass spectrometry (MALDI-TOF/MS) or MALDI-TOF/TOF MS to obtain peptide mass fingerprints of protein digests of proteins individually isolated from 2-D gel electrophoresis (Degrelle *et al.*, 2009). Later, with the use of the Liquid Chromatography coupled to MS (Dongre *et al.*, 1997) the proteomics strategies known as shotgun or bottom-up approaches became a valuable tool for high complex and large-scale protein analysis. They are non-gel based strategies, thus avoiding the work-intensive, time-consuming and less efficient 2-D electrophoresis-based proteomics. Shotgun proteomics is equivalent to shotgun genomic sequencing, in which the DNA is fragmented, sequenced, and overlapped to "reconstruct" the whole sequence. Shotgun proteomics is based on LC-MS/MS, and is considered currently the leading proteomics technology. Wolters *et al.* (2001) also introduced the MudPIT (multidimensional protein identification technology) approach for 2-D LC-MS/MS proteomics. In MudPIT, the tryptic digests of all proteins contained in a biological sample (e.g. a cell lysate) are separated according to acidity in the first LC run and hydrophobicity in the second LC run. The 2D-LC eluted peptides are submitted then to ESI-MS/MS thus producing a set of fragment ions. The *m/z* values of these fragment ions correlated to their 2D-LC retention times allow the identification of individual proteins in the sample. MudPIT has been shown to identify a greater number of proteins as compared to traditional gel-based MS approaches (Kline and Wu, 2009; Richardson *et al.*, 2009).

Usually, proteomics data is used to compare different states of a specific organ or the whole organism, such as a healthy *versus* a diseased tissue. Quantitation is therefore a must in such comparative studies, and the power of MS-proteomics for quantification has increased dramatically by the use of isotope labeling (Zhu *et al.*, 2010). Amino acids (AA) possessing light or heavy isotopes (as ^{13}C or ^{15}N) will behave rather similarly in biological systems and in sample preparation (tryptic digestion and LC), but the mass spectrometer, as discussed, is able to separate, measure and quantify each light and heavy isotopologues of the same molecule. Major isotope labeling MS-proteomics strategies are known as ICAT (isotope coded affinity tags), iTRAQ (isobaric tags for relative and absolute quantification), and SILAC (stable-isotope labeling by AA in cell culture; Gingras *et al.*, 2007).

Stable-isotope strategies allow confident protein quantification, but "label-free" methods are also emerging as attractive alternatives. These strategies involve the redundant peptide-counting for estimating the abundance of a given protein in a LC-MS/MS run. MS-based proteomics can also be used for protein post-translational modifications (PTM) studies. PTM play pivotal roles in protein activity regulation, and PTM quantification provides a better understanding of disease mechanisms, cell differentiation and pluripotency, and facilitates the discovery of biomarkers for molecular classification of disease and cell differentiation stages (Pan *et al.*, 2009).

For instance, using 2D-gel electrophoresis followed by proteins isolation and digestion, a comprehensive profile of the abundant proteins of ovoid (at day 11 of gestation) and elongating (at day 12 of gestation) pig embryos was performed (Degrelle *et al.*, 2009). After MALDI-TOF MS and LC-MS/MS analysis of 305 isolated spots, 275 proteins were identified and 174 of them were found to be distinct. Differentially up-regulated proteins spanned a variety of functional categories, such as heat-shock proteins, translational factors, metabolic enzymes, cytoskeletal elements, and signal transducers, which helped to better comprehend biological mechanisms underlying the crucial stage of elongation in pig conceptuses. MS-proteomics analysis in embryos has been recently reported for porcine (Sutovsky *et al.*, 2005), mouse (Katz-Jaffe *et al.*, 2005) and human embryos (Katz-Jaffe *et al.*, 2006a, b),

bringing new information regarding protein profile as a function of oxygen concentration during embryo *in vitro* culture and between different stages of development. In assisted reproductive technologies (ART), the proteome is of particular interest in non-invasive gamete and embryo assessment by means of the secretome, e.g. those proteins produced by cells and secreted in the surrounding medium (Hathout, 2007). Potential protein biomarkers in the secretome of human embryos have also been identified and are under validation (Katz-Jaffe *et al.*, 2009).

Recently Mullen *et al*. (2012) studied the protein composition of the histotroph during the preimplantation stage in cattle using a LC-MS/MS proteomic approach. They could identify 300 proteins on day 7 and 510 proteins on day 13. The functional analysis of the 34 differentially expressed proteins (including 14 novel to histotroph) revealed distinct biological roles putatively involved in early pregnancy, including remodeling of the uterine environment in preparation for implantation, nutrient metabolism, embryo growth, development and protection, maintenance of uterine health and maternal immune modulation (Mullen *et al.*, 2012). Using a quantitative ITRAQ proteomic approach Faulkner *et al*. (2012) identified and quantified the proteins present in plasma and uterine flushings from beef heifers on day 7 of the estrous cycle. With this approach they could observe a total of 35 proteins that were present in higher and 18 that were present in lower in uterine flushings including metabolic enzymes, proteins with anti-oxidant activity and those involved in modulation of the immune response.

Metabolomic analyses

Cells produce a myriad of metabolites, which are the functional end products of all biological processes, and therefore a promising form of non-invasive methods for embryo selection in the ART field. Metabolomic analysis approaches are aimed at identifying and quantifying target intracellular and extracellular metabolites usually with MW lower than 1.500 Da. Metabolome data involve the variability at the atomic level present in small molecules, making it more complex than genomics (made up from four bases) and proteomics (made up from 22 amino acids). To address this diversity, the metabolome can be subdivided into smaller omics subgroups (e.g., lipidomics, glycomics and peptidomics). The tremendous amount of information generated by metabolome analysis has been tackled with increasing efficacy by advances in bioinformatics. Compared to transcriptomics and proteomics, metabolomics is also considered a superior measure of cellular activity, especially for oocytes and embryos, since mRNA levels may fail to correlate with increased protein expression, and proteins may not be enzymatically active (Gygi *et al.*, 1999; Singh and Sinclair, 2007).

Traditionally, for metabolomic analysis, techniques such as nuclear magnetic resonance (NMR) and Fourier transform infrared spectroscopy (FTIR; Katz-Jaffe *et al.*, 2009) have been used. Nonetheless, due to the unmatched combination of speed, sensitivity and selectivity, MS approaches are becoming the most important techniques in current metabolomics studies. Compared to NMR, MS metabolomics presents higher sensitivity, higher-throughput and better identification power for unknown molecules in samples, with greater simplicity by eliminating or minimizing sample workup, pre-separation and chemical manipulation. For highly complex mixtures, coupling to LC may be required. Gas chromatography coupled to mass spectrometry (GC-MS and GC-MS/MS) is another approach used in this field (Budczies *et al.*, 2012). Target analysis or metabolite profiling are the basic strategies of metabolomics. Targeted analysis focuses usually on specific pathways and involves quantitative analysis. Metabolite profiling is the scanning of a large number of intracellular metabolites under a defined condition. Metabolite footprinting has been proposed as the measurement of all extracellular metabolites present in culture medium samples, which have been secreted by cells or organisms (Allen *et al.*, 2003; Villas-Boas *et al.*, 2005). High-resolution mass spectrometers have also been used for metabolite screening, especially lipids, in different biological matrices. This approach allows the exact molecular mass determination of compounds injected in the mass spectrometer. Coupling the LC technique to a high-resolution MS allows for the determination of a large number of metabolites in biological matrices and is the best for shotgun and untargeted lipidomics.

In a pioneer study carried out by our laboratory (unpublished data) uterine flushings and endometrial fragments of the uterine horn ipsilateral to the corpus luteum were assessed for their oxylipin composition, meaning the determination of absolute concentrations of unsaturated fatty acid oxidative metabolites (e.g., eicosanoids). Animals were synchronized and induced to ovulate following the model described earlier, in which low postovulatory progesterone cows (LP) ovulate smaller follicles and produce less progesterone, and high postovulatory progesterone cows ovulate larger follicles and produce greater amounts of progesterone (HP). Samples were collected on day 7 after induction of ovulation. Concentrations of the 87 different oxylipins were measured simultaneously using a liquid chromatography mass spectrometry (LC-MS) approach in both flushings and endometrium. The sum of all compounds within each precursor group was compared between HP and LP. In addition, the ratio of summed concentrations among precursor groups were compared between HP and LP groups. Whereas total concentration per precursor group (e.g., arachidonic acid, AA; linoleic acid, LA; dihomo-gamma-linolenic

acid, DHGLA; alpha-linolenic acid, ALA; eicosahexapentaenoic acid, EPA; docosahexaenoic acid, DHA) was not different in flushings, the ratios between total concentration of precursors for DHGLA:EPA, DHGLA:ALA and DHGLA:AA tended to be reduced in HP *vs*. LP (0.1 ± 0.03 *vs*. 0.23 ± 0.07, 0.13 ± 0.04 *vs*. 0.3 ± 0.11, and 0.008 ± 0.002 *vs*. 0.01 ± 0.002, respectively; $P < 0.08$). No differences were observed in the oxylipin profile of endometrial tissue. It was, therefore, postulated that manipulation of preovulatory follicle growth and consequent changes in the endocrine milieu during the first week of diestrus influences the oxylipid profile of uterine flushings, which may be associated with pregnancy outcome. No other metabolomics studies of the uterine environment in cattle have been reported to this date.

Systems Biology analytical approaches

All "omics" techniques are encompassed in the HDB approach, which refers to the simultaneous study of the different biological levels of an organ, tissue or an organism. The fundamental premise of HDB is that the evolutionary complexity of biological systems renders them difficult to comprehensively understand using only a reductionist approach, studying just units of the whole. An improved way of trying to understand a biological system is to acquire the greatest number of measurements of different variables in the different levels of the system. This approach can be achieved by the use of HDB. Nowadays it became clear that it is not individual genes, but rather, whole biological pathways and networks that drive an organism's response to a wide range of stimuli and the development of the range of phenotypes we observe. Many biologically significant networks, including metabolic networks, signal transduction networks and transcriptional regulatory networks, among others are involved in practically all what happens in the cells. In this context, the HDB approach can provide interesting results and help to understand the complexity of biological systems.

Certainly, between all the terms used in the "omics" field, Systems Biology is the most challenging to explain. Systems Biology attempts to integrate the different "omics" techniques to produce an holistic view of the function of a cell, tissue, organ or organism. Basically, it is a biology-based interdisciplinary study field that focuses on complex interactions in biological systems. Systems Biology seeks to understand how system properties emerge from the non-linear interactions of multiple components (Kitano, 2002a, b; Aderem, 2005; Diez *et al.*, 2010). The connections and interactions between individual constituents including genes, proteins, and metabolites are examined at the level of the cell, tissue, and organ to ultimately describe the entire organism or system (Barabasi and Oltvai, 2004; Ahn *et al.*, 2006). The intent is to identify the biological networks that connect the differing system elements, thereby defining the characteristics that describe the overall system. This information can then be used to derive mechanistic information on biological processes as well as identify potential target sites for therapeutic intervention (Hood *et al.*, 2004). In the opposite side of the reductionist science, where the scientists try to explain different small parts of the hole, Systems Biology integrate the same idea of HDB approach. In the last few years several papers and reviews about this theme have been published (D'Alexandri, 2010). Once again, no publications using systems biology approaches to study the uterus are available to this date.

Conclusions

In general, the peri-implantation period of gestation accounts for a major portion of economic losses associated with reproduction in cattle. Coincidentally, the biology of processes occurring during this period is poorly understood. It is our view that the complexity of the uterine environment during the preimplantation period must be researched using holistic approaches, such as the Systems Biology approach. Such an approach relies on a quiver of modern tools that are just starting to be used to study the uterus. Probing the uterine environment using Systems Biology tools will provide the basic information necessary to understand, diagnose and treat the preimplantation pregnancy losses in cattle.

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